Deficiency of the multi-copy mouse Y gene Sly causes sperm DNA damage and abnormal chromatin packaging.

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Running title: Sperm DNA damage in shSLY mice

Keywords: sperm DNA damage, chromatin remodeling, Y chromosome, male infertility
Summary

In mouse and man Y chromosome deletions are frequently associated with spermatogenic defects. Mice with extensive deletions of non-pairing Y chromosome long arm (NPYq) are infertile and produce sperm with grossly misshapen heads, abnormal chromatin packaging, and DNA damage. The NPYq-encoded multi-copy gene *Sly* controls the expression of sex chromosome genes after meiosis and *Sly* deficiency results in a remarkable upregulation of sex chromosome genes. *Sly* deficiency has been shown to be the underlying cause of the sperm head anomalies and infertility associated with NPYq gene loss, but it was not known whether it recapitulates sperm DNA damage phenotype. Here, we produced and examined mice with transgenically (RNAi) silenced *Sly* and demonstrated that these mice have increased incidence of sperm with DNA damage and poorly condensed and insufficiently protaminated chromatin. We also investigated the contribution of each of the two *Sly* encoded transcript variants and noted that the phenotype was only observed when both variants were knocked-down, and that the phenotype was intermediate in severity compared to mice with severe NPYq deficiency. Our data demonstrate that *Sly* deficiency is responsible for the sperm DNA damage/chromatin packaging defects observed in mice with NPYq deletions and point to SLY proteins involvement in chromatin reprogramming during spermiogenesis, likely through their effect on the postmeiotic expression of spermiogenic genes. Considering the importance of sperm epigenome for embryonic and fetal development and possibility of its inter-generational transmission, our results are important for future investigations of the molecular mechanisms of this biologically and clinically important process.
Introduction

Deletions of the Y chromosome are frequently associated with spermatogenic defects both in mice and in humans. In mice, the male specific, non-pairing Y chromosome long arm (NPYq) encompasses ~90% of the Y-specific DNA content and comprises mostly repetitive sequences including multiple copies of at least 4 distinct genes that are expressed in spermatids: Styl1/2, Sly, Asty, Orly (Toure et al., 2005). These genes show a progressive reduction in transcript levels with increasing NPYq deficiency and are candidates for contributing to the sperm defects associated with NPYq deletions. Mice with severe NPYq deletions are infertile in vivo and in vitro and have all sperm with head-shape defects (Burgoyne et al., 1992). Live offspring were obtained from the infertile males when intracytoplasmic sperm injection (ICSI) was used (Ward and Burgoyne, 2006; Yamauchi et al., 2009), but low efficiency of assisted reproduction suggested that sperm impairment reached beyond their inability to transmit the paternal genome to the oocyte in vivo, and might have involved DNA changes.

In support of this notion we have recently shown that sperm from mice with severe NPYq deficiencies had DNA damage and abnormal chromatin packaging (Yamauchi et al., 2010). Epididymal sperm from mice with severe NPYq deficiency (i.e. deletion of nine-tenths or the entire NPYq gene complement) were impaired in oocyte activation ability following ICSI, and there was an increased incidence of oocyte arrest and paternal chromosome breaks. Comet assays revealed higher DNA damage in both epididymal and testicular sperm from these mice relative to controls, with epididymal sperm the more severely affected. Moreover, epididymal sperm from mutant mice also suffered from impaired membrane integrity, abnormal chromatin condensation and suboptimal chromatin protamination, making it likely that the increased DNA damage associated with NPYq deficiency is a consequence of disturbed chromatin remodeling during spermiogenesis.

Sly (Sycep3-like Y-linked) is present on NPYq in at least 100 copies, 70 of which retain an open reading frame, and encodes a protein that is highly expressed in round spermatids, with nuclear localization at spermiogenesis steps 2/3 to 9 (Cocquet et al., 2009). The SLY protein contains a conserved COR1 domain implicated in chromatin binding (Ellis et al., 2005). The characterization of ‘shSLY mice’, in which Sly expression has been knocked-down by transgenically-delivered short hairpin RNAs, showed that Sly deficiency is the major underlying
cause of the sperm head anomalies and infertility associated with NPYq deficiency (Cocquet et al., 2009). This study preceded our finding of chromatin packaging defects and increased DNA damage in NPYq deficient mice (Yamauchi et al., 2010), and it is therefore not known if shSLY mice recapitulated this aspect of NPYq deficient phenotype.

In the present study we tested the hypothesis that Sly deficiency is responsible for the sperm DNA damage/chromatin packaging defects observed in mice with NPYq deletions, and established Sly transcript requirements for this phenotype.

**Results**

**Characterization of a new shSLY line in which Sly1 but not Sly2 expression is knocked down**

The NPYq specific gene Sly encodes two transcript variants, Sly_v1 and Sly_v2 (Reynard et al., 2009), hereafter called Sly1 and Sly2 (Fig. 1A). Sly1 is a full-length isoform and encodes a ~40 kDa protein (referred to as SLY1), detected by our anti-SLY1 antibody (Reynard et al., 2009). Exons 5–6, which arose from a duplication of exons 3–4 (Ellis et al., 2007), are spliced out in the Sly2 isoform. Our anti-SLY1 antibody does not detect SLY2 protein but Sly2 can be translated: a transgene-derived SLY2 protein fused to Flag tag was detectable by an anti-Flag antibody (supplementary material S1). We previously reported that, on an MF1 background, transgenic delivery of Sly-specific short hairpin sh367 RNAs led to significant decrease of Sly RNA and protein levels, with both splice variants being affected (Cocquet et al., 2009). These Sly-deficient males presented severe sperm head abnormalities and reduced fertilizing abilities. Here, we characterized a new line, sh344 (Fig. 1A,B), which was not reported on earlier. As in line sh367, expression of sh344 RNA was associated with a decrease of global Sly transcript level and of SLY1 protein level in the testes: sh367 mice showed 75% reduction of global Sly (Sly1+2) and 90% reduction of Sly1 transcripts while in sh344 the reduction was ~50% of Sly1+2 and ~80% of Sly1 transcripts (Fig. 2A and supplementary material S2A). No SLY1 protein was detected in sh367 testes by western blotting even with prolonged exposure while in sh344 SLY1 protein level was decreased by 85% (Fig. 2B,C).

Because exons 3-4 are very similar to exons 5-6 (~86% identity, Fig. 1C) we failed to design real time PCR primers that were specific to Sly2 transcripts. We therefore quantified the expression of Sly1 and Sly2 transcripts independently with semi-quantitative RT-PCR, using primers, which
span the alternatively spliced exons 5-6. These experiments demonstrated that, in line sh344, 
*Sly1* but not *Sly2* transcripts were knocked down, while both *Sly1* and *Sly2* transcripts were 
knocked down in line sh367 (Fig. 2D and supplementary material S2C). Alignment of *Sly2* 
transcript sequence with sh344 and sh367 RNA sequences shows the presence of two 
mismatches with sh344 sequence while there is only one with sh367 sequence (Fig. 1B). Due to 
the lack of an SLY2-specific antibody, we could not measure/observe SLY2 protein level, but 
one can assume that SLY2 level mimics that of *Sly2* transcript and, as a consequence, SLY2 
protein level is expected to be unchanged in sh344 testes but decreased in sh367 testes. 
These expression analyses demonstrate that, on a predominantly C57BL/6 background, sh367 is 
deficient for both *Sly1* and *Sly2* (i.e. *Sly*-deficient males) while sh344 line is only deficient for 
*Sly1* (i.e. *Sly1*-only deficient males). To determine the impact of each *Sly* variant on sperm 
differentiation, we analyzed and compared the testicular phenotypes of these two lines.

**Sly-deficient but not *Sly1*-only deficient males have increased sperm headshape defects 
and impaired sperm function in vitro**

*Sly*-deficient sh367 mice were previously shown to have impaired sperm function on MF1 
genetic background (Cocquet et al., 2009). We sought to determine if C57BL/6 sh367 mice and 
*Sly1*-only deficient mice (i.e. sh344) also presented impaired sperm function. When we assessed 
fertility *in vitro*, sperm from sh367, but not from sh344 mice, had significantly impaired ability 
to fertilize oocytes and those oocytes that were successfully fertilized exhibited poorer 
development to blastocyst (Fig. 3A). This sperm dysfunction was not related to decrease in testis 
size, decrease in cauda epididymal sperm number or insufficient sperm motility, which were all 
normal and comparable between both *Sly* and *Sly1*-only deficient lines and controls. Detailed 
cauda epididymal sperm headshape analysis differentiating between sperm that were normal, 
slightly deformed, and grossly deformed, demonstrated that in *Sly*-deficient sh367 mice virtually 
all sperm were morphologically abnormal, and had gross head shape defects. However, *Sly1*- 
only deficient mice were not significantly different from non-transgenic siblings, both having 
>85% of morphologically normal sperm (Fig. 3B).

To summarize, sperm analyses point to severe sperm headshape defects and impairment in the 
ability of sperm to fertilize oocytes in vitro in *Sly*-deficient, but not in *Sly1*-only deficient mice.

**Sly-deficient but not *Sly1*-only deficient males have increased sperm DNA damage**
We have previously shown that testicular sperm from normal wild-type mice have more comet assay detectable sperm DNA fragmentation than cauda epididymal sperm, and that freezing increases DNA damage in sperm from both sources; in mice with severe NPYq deficiencies this sperm DNA fragmentation is markedly increased with epididymal sperm more affected than testicular sperm, and with both types of sperm more susceptible to freezing (Yamauchi et al., 2010). Here, we tested fresh and frozen cauda epididymal and fresh and frozen testicular sperm from *Sly*- (sh367) and *Sly1*-only (sh344) deficient transgenic lines, with their negative siblings as controls.

As in our previous study, because of the non-normality of the underlying data, we have used male means for the analysis. A preliminary ANOVA analysis revealed no significant differences between sh344 and sh367 negative siblings, so the control data were pooled. We then carried out ANOVA analysis of sh344, sh367, and pooled controls, with genotype, sperm source (testis or epididymis) and sperm status (fresh or frozen) as factors (Fig. 4A,B,C). As in controls described in our previous study, in all genotypes there was a significant (P<0.000001) effect of sperm source (testicular sperm more affected than epididymal, Fig. 4B) and sperm status (frozen sperm more affected than fresh sperm, Fig. 4C). There was also a significant effect of genotype (Fig. 4A, P=0.0004) with sh367 mice, but not sh344 mice, having significantly (P=0.00009) longer comet tail lengths than controls. The histograms of the raw data for each sperm type shed light on the nature of sperm DNA damage revealed by the ANOVA analysis (Fig. 4D). In the controls the baseline is provided by fresh epididymal sperm, in which 99% (792/800) of sperm fall in a normally distributed population of sperm with comet tails in the range 41-188 μm, and the remaining 1% (8/800) with comet tails ranging from 210 to 308 μm, indicating severe sperm DNA fragmentation. Therefore, as a yardstick for increases in sperm DNA damage for the remaining distributions we have calculated the percentage of sperm with comet tail lengths exceeding 200 μm. Freezing the epididymal sperm shifts the entire distribution a little to the right, and increases the proportion of sperm with severe DNA damage; as a consequence 9% (69/800) of sperm now have comet tail lengths exceeding 200 μm. Comparison of distributions of fresh testicular sperm with fresh epididymal sperm (14%, 113/800 vs. 1%, 8/800 >200 μm) indicates a more marked shift of the distribution to the right but a less marked increase in sperm with the highest DNA damage. In agreement with the ANOVA analysis, sperm in sh367 mice are more affected than in controls resulting in further increases in the proportion of sperm with...
comet tails >200 µm in all categories except fresh testis; nevertheless, for fresh testis it is apparent that the ‘normal’ part of the distribution has moved to the right which is consistent with the ANOVA result (Fig. 4D).

Next, we compared Sly-deficient mice with mice with moderate (2/3NPYq-) and severe (NPYq-2) NPYq deficiency (Fig. 4E,F,G). The controls in this comparison were negative siblings of sh344 and sh367 mice and wild-type controls of NPYq deficient mice; the controls did not differ from each other and were therefore pooled. ANOVA analysis demonstrated a significant (P<0.000001) effect of genotype (Fig. 4E) with sh367 and NPYq-2, but not 2/3NPYq- and sh344, mice significantly different from controls. In both Sly and NPYq deficient mice, there was a significant effect of sperm status (Fig. 4F; P<0.000001) with frozen sperm having significantly longer comet tail lengths than fresh; the same effect was seen in controls. However, while sh344, sh367 and control mice had overall longer comet tail lengths in testicular sperm than in epididymal sperm, this was not true for NPYq-2 mice, in which epididymal sperm were more affected (Fig. 4G; genotype/source interaction P=0.0007).

Overall, comet assay analysis points to Sly-deficient but not Sly1-only deficient mice having an increased susceptibility to sperm DNA damage. However, this DNA damage is less severe than in mice with complete NPYq deficiency, and is not preferentially affecting epididymal sperm as was observed in severely NPYq deficient mice.

**Sly deficiency is not associated with impairment of early fertilization steps after ICSI and does not lead to increased incidence of paternal chromosome breaks in the zygotes.**

We have previously shown that when cauda epididymal sperm from mice with severe NPYq deficiencies were injected into the oocytes by ICSI, there was an impairment in oocyte activation, increased incidence of oocyte arrest at the pronuclei stage, and presence of paternal chromosome breaks in the zygote (Yamauchi et al., 2010). Here, following the design of the previous study, we injected fresh and frozen cauda epididymal and fresh and frozen testicular sperm from Sly- (sh367) and Sly1-only (sh344) deficient mice (and transgene negative siblings serving as controls), into the oocytes and examined early post-fertilization steps.

No significant differences between the same sperm types from Sly- and Sly1-only deficient mouse lines and controls, and between epididymal and testicular sperm regardless of whether sperm were fresh or frozen ('within genotype' comparison) were observed in incidence of oocyte activation, oocyte arrest, and oocytes with normal paternal chromosomes (supplementary
material S3). We also carried out a single analysis of Sly-deficient mice and controls by ANOVA with genotype, sperm source (testis or epididymis) and sperm status (fresh or frozen) as factors (data not shown). In all measured endpoints there was no effect of genotype.

We then placed the data obtained with sh367 and sh344 mice in perspective of data obtained earlier with NPYq deficient mice (Yamauchi et al., 2010) (supplementary material S4), pooling the data obtained after ICSI with four types of sperm, and using as a control pooled controls from both studies. Single ANOVA analysis demonstrated a significant (P<0.001) effect of genotype, with sh367, sh344, and 2/3NPYq- mice being no different than controls and significantly less affected than mice with severe NPYq deficiency (9/10NPYq- and NPYq-2) in incidence of arrested oocytes (S4A), oocytes with normal paternal chromosomes (S4B), and aberration rate (S4C).

To assess whether lack of 'ICSI phenotype' in Sly-deficient mice was due to milder sperm headshape defects and unintentional preselection during injection, we compared sh367 frozen cauda epididymal sperm purposely preselected according to their headshape into two groups: sperm with slight (categories S1-2 shown in Fig. 3) and gross (G4-6 shown in Fig. 3) headshape defects (supplementary material S5). No differences in the incidence of oocytes activated, arrested, and with normal paternal chromosomes were noted after injection of sperm with slight versus gross headshape defects (Fisher's Exact Test).

Overall, ICSI data imply that contrary to what is seen in mice with severe NPYq deficiency, Sly knockdown does not impair sperm function in assisted fertilization. Sperm from Sly-deficient mice activate oocytes normally yielding no chromosome damage in the ICSI-generated zygotes.

**Sly-deficient but not Sly1-only deficient mice have poor sperm chromatin condensation.**

Transmission electron microscopy was used to examine chromatin compaction in cauda epididymal sperm from sh367 Sly-deficient mice, sh344 Sly1-only deficient mice, and their negative siblings as controls (Fig. 5). Sperm were categorized into three categories: those with properly condensed, slightly decondensed, and severely decondensed chromatin (Fig. 5A-C). In controls and sh344 mice the vast majority of sperm had properly condensed chromatin (87% and 77%); less than 1% of sperm had severely decondensed chromatin. In sh367 males, sperm with properly condensed chromatin were significantly less frequent (50%) and the incidence of sperm severely affected increased to 3.4%. Single ANOVA analysis comparing the incidence of sperm
with properly condensed chromatin in these three genotypes revealed that sh367 mice were significantly different from control (P=0.00002) and from sh344 (P=0.003) (Fig. 5D).

When the data obtained with $Sly$- and $SlyI$-only deficient mice were put in the context of data obtained before with sperm from mice with NPYq deficiencies (Yamauchi et al., 2010), single ANOVA analysis showed a significant effect of genotype (P<0.000001, Fig. 5E). All tested genotypes were significantly different than controls, which in this analysis were pooled controls from both studies. However, a clear gradation in chromatin condensation deficiency could be observed, with sh344 mice being similar to mice with a moderate NPYq gene loss (2/3NPYq-), sh367 mice being similar to those with extensive NPYq deletion (9/10NPYq-), and mice lacking the entire NPYq gene complement being most affected.

Overall, the data point to $Sly$-deficient but not $SlyI$-only deficient mice exhibiting impairment in sperm chromatin condensation.

**Mice with severe NPYq deficiency and Sly-deficient mice have impaired sperm chromatin protamination**

Poor chromatin condensation can result from insufficient DNA protamination. Chromomycin A3 (CMA3) is a fluorescent dye that intercalates into DNA but because of its size it can only bind to sperm DNA when it is not completely condensed and supposedly loosely bound to DNA binding proteins. Therefore, CMA3 staining has been used as an indirect measure of the level of protamination in sperm, in humans, mice and other species (Bianchi et al., 1993; Delbes et al., 2007; Meyer-Ficca et al., 2009; Noblanc et al., 2012; Simoes et al., 2009). Here, we used this approach to examine cauda epididymal sperm from $Sly$- and $SlyI$-only deficient mice and their negative siblings as controls (Fig. 6). Because CMA3 staining was not included in our previous analysis of NPYq deficient mice (Yamauchi et al., 2010), we also tested three NPYq deficient genotypes and their specific controls.

The proportion of CMA3 positive sperm was similar among the controls justifying pooling for the next comparisons. Single ANOVA analysis on pooled controls and all $Sly$ and NPYq deficient genotypes (Fig. 6B) revealed a significant effect of genotype (P<0.0001) with sh367 (P=0.0098), 9/10NPYq- (P=0.000013) and NPYq-2 (P<0.000001) having more CMA3 positive sperm when compared to controls. Mice with moderate NPYq gene loss (2/3NPYq-) and sh344 mice were not significantly different from controls, and were significantly less affected than the remaining three genotypes. The results from this multi-genotype ANOVA were further supported
by one-to-one ANOVA comparisons of each genotype with its specific control. In this analysis sh344 and 2/3NPYq- were not different than their specific controls, but sh367 (P=0.017), 9/10NPYq- (P=0.0007) and NPYq-2 (P=0.0001) were.

In support for CMA3 analysis we also examined protamine processing in cauda epididymal sperm from sh367 mice using acid-urea gel electrophoresis and western blot (supplementary material S6), following the strategy described earlier for NPYq deficient mice (Yamauchi et al., 2010). Separated sperm nuclear proteins corresponding to the same sperm number were assessed on Coomasie blue stained gel and by immunoblot with Hub2B and PreP2 antibodies recognizing mature and immature protamine P2 forms, respectively. The analysis was performed with two-way ANOVA, with gel and genotype as factors. We did not observe significant differences between Sly-deficient and control mice in the intensities of bands representing mature protamines. No preP2 bands originating from sperm were detected on any of Coomassie blue stained gels. When the gels were blotted with anti-preP2 antibody, bands were detected in 4 out of 5 Sly-deficient and 2 out of 5 control males. Although the difference in average preP2 band intensity did not reach significance, it was more than twice higher in sh367 mice.

Overall, mice with severe NPYq deficiency had significantly increased incidence of CMA3 positive sperm suggesting impaired protamination; Sly-deficient mice, but not Sly1-only deficient mice, exhibited the same phenotype but were less severely affected. Impaired protamination in sperm from Sly-deficient mice was also shown directly using nuclear protein analysis but similarly as in CMA3 analysis, it was much less severe than that noted before for mice with severe NPYq deficiency (Yamauchi et al., 2010).

**Sly1-only deficient males have a minor de-repression of X and Y encoded spermiogenic genes compared to global de-repression in Sly-deficient males**

Sly-deficient males display a remarkable upregulation of sex chromosome genes after meiosis and we have proposed that the multiple defects associated with Sly deficiency (headshape malformation, impairment of sperm function, etc.) may likely a consequence of the massive and global upregulation of spermiogenic genes (Cocquet et al., 2009). Since Sly1-only deficient males do not display any of the above-mentioned defects we have analyzed the level of expression of several X and Y spermiogenic genes. Results presented in Fig. 7 show a derepression of some of the X and Y encoded genes analyzed in Sly1-only deficient males but this derepression is significantly less pronounced than in Sly-deficient males. Microarray analysis
supplementary material S7) validated the qRT-PCR data, with sh344 males showing the same widespread overexpression of X-linked spermatid genes previously seen in sh367 (Cocquet et al., 2009), but to a much lesser extent.

**Discussion**

Even relatively minor errors in chromatin remodeling during spermiogenesis are associated with sperm DNA damage and infertility, yet little is known about the etiology. Mice with severe NPYq deletions are infertile due to severe sperm differentiation defects (Ward and Burgoyne, 2006; Yamauchi et al., 2009). We have recently observed that sperm from these mice presented abnormal chromatin packaging and DNA damage. Moreover, when these sperm were injected into the oocytes, a significant increase of oocyte arrest at pronuclei stage and of chromosome aberrations in the fertilized eggs were noted (Yamauchi et al., 2010). Here we provide evidence that the deficiency of NPYq encoded gene *Sly* is associated with sperm DNA damage and poor sperm chromatin condensation, and propose that SLY plays a role in spermatid-specific chromatin remodeling.

How can *Sly*/SLY be involved in sperm DNA damage phenotype? SLY protein has been shown to control the postmeiotic expression of >100 genes, the majority of which are encoded on sex chromosomes. In *Sly*-deficient males, the repressive epigenetic marks normally associated with the sex chromosomes (i.e. CBX1 and H3K9me3) are decreased, which leads to the upregulation of many X- and Y-encoded spermiogenic genes (Cocquet et al., 2009). The multiple spermiogenesis defects associated with *Sly* deficiency, including the sperm DNA damage and abnormal chromatin packaging described in the present study, are likely a consequence of this massive change in spermiogenic gene expression. In *Sly1*-only deficient males, there are no clear defects of the spermiogenesis function, and the upregulation of spermiogenic X and Y genes is very limited. In all probability, a certain threshold of upregulation must be reached to significantly alter sperm differentiation and function.

In theory, the sperm differentiation defects observed in *Sly*-deficient and NPYq-deficient mice could result from the deregulation of any of the >100 deregulated genes, or from the collective deregulation of several of them. Nevertheless, several genes appear to be promising individual candidates. For instance, alteration in the expression of *H2a1l* and *H2a2l2y* genes (Ferguson et al., 2009), which encode spermatid specific histone variants (Govin et al., 2007), could affect sperm
chromatin protamination and therefore spermiogenesis. A few autosomal genes were also
downregulated in *Sly*-deficient spermatids (Cocquet et al., 2009). Among them, the gene *Chaf1b*
was a very likely candidate to explain sperm DNA damage since it encodes a component of the
Chromatin Assembly Factor 1 (CAF-1), which is involved in the DNA damage and repair
process (Groth et al., 2007). RNAi silencing of the CHAF1B in proliferating human cells led to
accumulation of double strand DNA breaks, increased level of phosphorylated histone H2AX,
and ultimately cell death (Nabatiyan and Krude, 2004).

*Sly* genes are estimated to be present in between 70 to 100 copies in the mouse genome; the
majority of these encode two transcript splice variants, *Sly1* and *Sly2* (Cocquet et al., 2009; Ellis
et al., 2011; Scavetta and Tautz, 2010). In the present study, we have produced and characterized
a *Sly1*-only deficient mouse model, and shown that, in contrast to *Sly*-deficient mice, in which
both transcript variants are affected, *Sly1*-only deficient mice do not have sperm differentiation
defects. From our analyses, we can draw two hypotheses to explain the phenotypic differences
between those two models. *Sly2* may be the important functional variant and its knockdown
would be solely responsible for the derepression of spermiogenic genes and the various
subsequent sperm defects observed in *Sly*-deficient mice, or it is the global reduction of both *Sly*
variants that matters. Several arguments can be brought forward against the former scenario.

First, SLY1 protein co-localizes with the loci that are deregulated in *Sly*-deficient males
(Cocquet et al., 2009) and, therefore, is likely to play a role in the regulation of these loci.
Second, SLY1 protein structure is very similar to that of SLY2. The COR1 domain, which is
presumed to mediate chromatin binding, is identical in SLY1 and SLY2 proteins. SLY1 only
differs from SLY2 in the 34 additional amino acids encoded by exons 5-6. Since exons 5-6 arose
from a duplication of exons 3-4, the protein region encoded by exons 5-6 shares 70% identity
and 79% similarity with the protein region encoded by exon 3-4 (Fig. 1C). All in all, it is more
plausible that *Sly1* and *Sly2* genes encode proteins of similar function, and that the phenotype is
correlated with the global level of knockdown of *Sly1* and *Sly2*. Our *Sly*/SLY level quantification
on whole testes confirms this assumption, with a lower reduction of global *Sly* (*Sly1+2*)
transcript level and retention of some of SLY1 protein in phenotypically normal *Sly1*-only
deficient males, and a higher reduction of global *Sly* (*Sly1+2*) and no detectable SLY1 in
affected *sh367 Sly*-deficient males (Fig. 2A,C and supplementary material S2A).
In respect to sperm chromatin packaging/DNA damage, Sly-deficient mice recapitulate the NPYq-deficient phenotype only very moderately and much less significantly than other phenotypic features (Table 1). Moreover, Sly deficiency is not associated with impairment of early fertilization events after ICSI and does not lead to increased incidence of paternal chromosome breaks in the zygotes that are present in severely NPYq-deficient mice (supplementary material S3 and S4), and this is not due to preselection of sperm with milder headshape defect (supplementary material S5). The difference in Sly transcript levels between mice with severe NPYq gene loss, i.e. deletion removing nine-tenth (9/10NPYq-, 4%) or the entire NPYq (NPYq-2, 0%), and Sly-deficient sh367 mice (25%, supplementary material S2A) could explain the differences in the phenotype in these mice. However, lack of detectable SLY1 protein in all genotypes and similar Sly transcript levels in Sly-deficient and 9/10NPYq- mice (10% both) put the dependence of the phenotype exclusively on Sly/SLY levels in question. Mice with severe NPYq deficiency are lacking other genes encoded on the Y long arm, which could contribute to spermiogenic defects in these mice. It therefore cannot be excluded that these other NPYq genes, which are not only present but also upregulated in Sly-deficient mice (Cocquet et al., 2009; Ellis et al., 2005), mitigate the sperm chromatin packaging/DNA damage phenotype and/or are involved in early embryonic events; either directly or indirectly through the regulation of other loci. The most plausible gene candidate would be Ssty1/2 (Spermatid-specific transcripts Y-encoded), which is present on the mouse NPYq in more than 100 copies, is specifically transcribed in the testis in round spermatids, and is the only other NPYq gene that is translated. Ssty1/2 belongs to the Spindlin family, one member of which has recently been shown to bind to methylated histone H3 (Wang et al., 2011); Ssty1/2 could therefore contribute to chromatin remodeling during spermiogenesis, along with Sly. Future work utilizing Sly and/or Ssty1/2 transgene additions to severely NPYq-deficient mice should help clarifying the contribution of Sly and Ssty1/2.

What are the clinical implications of our work? Infertility is a major public health problem that affects ~10%, or 72.4 million couples worldwide (Boivin et al., 2007). Male factor infertility contributes to at least half of infertility cases, and its primary cause is aberrant spermatogenesis. Packaging of the haploid genome into the sperm head requires extensive chromatin remodeling, during which histones are disassembled and replaced with protamines. Deficiencies in protamination that leave portions of DNA poorly compacted are associated with sperm DNA...
damage; in humans this is a risk factor for adverse clinical outcomes including poor fertilization, impaired development of the preimplantation embryo, miscarriage, and an increased risk of morbidity in the offspring (Lewis and Aitken, 2005). Recent evidence suggests that the remodeling of sperm chromatin leaves a significant epigenetic imprint in the form of retained histones and histone modifications (Brykczynska et al., 2010; Hammoud et al., 2009), and that disturbance of this epigenetic imprint during the remodeling could have clinically important consequences for offspring (de Boer et al., 2010; Ng et al., 2010). Indeed, a recent report on inter-generational paternal transmission of an acquired trait (impaired glucose-insulin homeostasis) in rats (Ng et al., 2010), which must have been mediated by an alteration of the epigenetic blueprint in sperm (Skinner, 2010), emphasizes the importance of the sperm epigenome for subsequent embryonic development.

All in all, a growing number of reports suggest that impairment of the sperm epigenome could have an impact on male fertility and on the offspring’s health; but the factors that lead to the retention of histones and their epigenetic marks in sperm are poorly understood. Our identification of Sly and its target genes as candidate chromatin remodeling regulators opens the door for future investigations of this clinically important process.

### Materials and methods

#### Chemicals

Mineral oil was purchased from Squibb and Sons (Princeton, NJ, USA) and equine (eCG) and human (hCG) chorionic gonadotrophin from Calbiochem (San Diego, CA, USA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated.

#### Plasmid generation and breeding of transgenic mice

Mice shSLY carry a transgene expressing short hairpin RNAs targeting the specific degradation of Sly transcripts. Two shSLY lines were analyzed in the present study: shSLY367 (or sh367), which was previously characterized on an MF1 genetic background (Cocquet et al., 2009) and shSLY344 (or sh344) (Fig. 1). A U6sh344 construct was produced using a PCR-based approach similar to that described in Harper et al (Harper et al., 2005) with the primers shown in supplementary material S8. The PCR product was cloned into the pCR2.1 vector and sequenced.
The U6sh344 cassette was then subcloned into pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA) using AflII restriction sites. The efficiency and specificity of the construct was checked by co-transfection as previously described for other U6shSLY constructs (Cocquet et al., 2009). The construct was then linearized by ApaL1 and BspH1, on-column purified, and microinjected into fertilized eggs from CBA/Ca6 x C57BL/10. Transgenic animals carrying the U6sh344 construct were identified by PCR (supplementary material S8). Both shSLY lines were backcrossed to C57BL/6, to make these mice comparable with NPYq deficient models used by us before for analysis of sperm DNA damage and chromatin remodeling (Yamauchi et al., 2010). shSLY males on a predominantly C57BL/6 (75-87.5%) genetic background were examined at 2-4 months of age. Non-transgenic siblings served as controls. Female B6D2F1 oocyte donors for IVF and ICSI (National Cancer Institute, Raleigh, NC) were used at 8-10 weeks of age. The mice were fed ad libitum with a standard diet and maintained in a temperature and light-controlled room (22ºC, 14h light/10h dark), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and National Research Council’s (NRC) “Guide for Care and Use of Laboratory Animals” (Council, 2011). The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

**Western blot**

Western blot analyses were performed as described previously (Reynard et al., 2009). Briefly, 10 to 15 µg of testis lysates were run on a 12% SDS/polyacrylamide gel. Following transfer and blocking, membranes were incubated overnight with a primary antibody (anti-SLY1 (Reynard et al., 2009) at 1/3,000 and anti-β actin at 1/50,000). Incubation with the corresponding secondary antibody coupled to peroxidase, and detection by chemiluminescence, were carried out as described by the manufacturer (SuperSignal West Pico, Pierce Biotechnology, Rockford, IL, USA). The analysis of sperm nuclear proteins using acid urea polyacrylamide electrophoresis (AU-PAGE) followed by western blot were done exactly as described by us earlier (Yamauchi et al., 2010).

**Real-time RT-PCR**

For real-time reverse transcription-polymerase chain reaction (RT-PCR), total testis RNA or RNA from round spermatids purified with STAPUT method (Bellve, 1993) was extracted using
Trizol, DNaseI-treated (Ambion, Austin, TX, USA), and purified using RNeasy Kit (Qiagen, Valencia, CA, USA). Reverse transcription of polyadenylated RNA was performed with Superscript Reverse Transcriptase III, according to the manufacturer’s guidelines (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SYBR Green PCR Master mix on an ABI StepOnePlus machine (Applied Biosystems, Carlsbad, CA, USA). PCR reactions were incubated at 95°C for 10 min followed by 35 PCR cycles (10 s at 95°C and 60 s at 60°C). For analysis of Sly expression, two types of PCR reactions were done: (1) ‘Sly1+2’ amplifying both Sly_v1 and Sly_v2 transcripts (Reynard et al., 2009) (primers Sly Global) and (2) ‘Sly1’ amplifying only Sly_v1 (primers Sly Long). All reactions were carried out in triplicate per assay, and β-actin or Acvrl (spermatid specific gene not regulated by Sly) was included on every plate as a loading control. The difference in PCR cycles with respect to β-actin or Acvrl (DCt) for a given experimental sample was subtracted from the mean DCt of the reference samples (non-transgenic siblings) (DDCt). For the quantification of Sly knockdown, values were further normalized to DDCt values of the β-actin or Acvrl. Expression analysis was also done for several X and Y encoded transcripts to test for their upregulation in shSLY mice. Primer sequences are shown in supplementary material S8.

Semi-quantitative RT-PCR

RT-PCR was performed as described for real-time RT-PCR except that Sly and β-actin were run separately and with cycle number decreased (26 for Sly and 24 for β-actin) to stop the reaction prior to saturation. The products were electrophoresed, Image J software (Rasband, 2007) was used to measure band intensity, and Sly1 and Sly2 values were normalized to β-actin. Primer sequences are listed in supplementary material S8.

Gamete collection and embryo culture

Female mice were induced to superovulate with injection of 5 IU eCG and 5 IU hCG given 48 h apart. Oocyte collection and subsequent oocyte manipulation, including microinjections, were done in HEPES-buffered CZB medium (HEPES-CZB, (Kimura and Yanagimachi, 1995)), with subsequent culture in CZB medium (Chatot et al., 1989) in an atmosphere of 5% CO2 in air. Sperm capacitation for IVF was done in T6 medium (Quinn et al., 1982). To obtain testicular sperm a portion of testis was cut off and minced in ETBS buffer (Kusakabe et al., 2001) or HEPES-CZB to release spermatogenic cells. To obtain epididymal sperm, caudae epididymides
were dissected and sperm were expressed with needles into HEPES-CZB, ETBS, T6 or PBS. Spermatozoa were allowed to disperse for 2-3 min at room temperature. The samples of testicular or epididymal cell suspension were used for in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), comet assay, transmission electron microscopy (TEM) analysis, chromomycin A3 (CMA3) staining, for isolation of protein for AU-PAGE, or were subjected to freezing.

Epididymal and testicular sperm freezing

Aliquots of 10 µl of cauda epididymal sperm or testicular cell suspension in ETBS were loaded in 0.25 ml straws (Edwards Innovation, Spring Valley, VA, USA). Each straw was sealed with Critoseal (Oxford Labware, St Louis, MO, USA) and placed in a plastic holder floating on the surface of the liquid nitrogen for 10 min before immersion. For thawing, the straws were removed from the storage container and immersed in a water bath at 37°C for 10 min and the contents were expressed into a Petri dish. Spermatozoa were used immediately for ICSI or other analyses.

In vitro fertilization (IVF) and embryo culture

Epididymal sperm were capacitated in T6 medium for 1.5 h at 37°C in a humidified atmosphere of 5% CO2. IVF was performed as previously described (Ajduk et al., 2006). Gametes were co-incubated for 4 h. After co-incubation, the oocytes were washed several times with HEPES-CZB, followed by at least one wash with CZB. Embryos were cultured in CZB and observed at different time-points for proper development; 24 h (2-cell stage), 48 h (4- or 8-cell stage), 72 h (morula or early blastocyst), 96 h (blastocyst).

Intracytoplasmic sperm injection (ICSI)

Intracytoplasmic sperm injection was carried out as previously described (Szczygiel and Yanagimachi, 2003), within 1-2 h from oocyte and sperm collection. Sperm-injected oocytes were transferred in CZB and cultured at 37°C. The survival and activation of injected oocytes was scored 1-2 h and 6 h after the commencement of culture, respectively. The oocytes with 2 well-developed pronuclei and extruded 2nd polar body were considered activated.

Sperm chromosome analysis
Chromosome preparation and analysis were performed as described by us before (Yamauchi et al., 2010). Activated oocytes were transferred into CZB containing 0.006 mg/ml vinblastine, which was added to inhibit spindle formation and syngamy. Between 19 and 21 h after ICSI, oocytes were treated with 1% pronase (1000 tyrosine units/mg; Kaken Pharmaceuticals, Tokyo, Japan) for 5 min at room temperature to soften the zonae pellucidae. Oocytes were then treated with hypotonic solution (1:1 mixture of 1% sodium citrate:30% fetal bovine serum) for 5 min at 37°C or 10 min at 25°C. Chromosomes were spread on glass slides by the gradual fixation/air-drying method (Mikamo and Kamiguchi, 1983). The preparations were stained with 2% Giemsa (Merck, Darmstadt, Germany) in PBS (pH 6.8) for 10 min for conventional chromosome analysis. The percentages of zygotes with normal and abnormal chromosomes were determined. In addition to scoring normal vs. abnormal karyoplates, we also calculated the incidence of chromosome aberrations, i.e. aberration rate, which represents the total number of aberrations divided by the number of oocytes examined. This aberration rate reflects the intensity of DNA damage. As chromosome aberrations we counted breaks, fragments, ring chromosomes, obvious translocations, and other structural chromosome defects detectable with conventional Giemsa staining.

**Comet assay**

Comet assay was performed using the Trevigen Kit (Trevigen, Gaithersburg, MD, USA, cat no 4250-050-K) under neutral conditions as described by us before (Yamauchi et al., 2007). For each sample tested, 100 DNA tails were photographed and analyzed per slide. The length of each tail was measured from the center of the comet to the end of the tail using Image J software (Rasband, 2007), and each tail was categorized into one of four tail types reflecting the severity of DNA damage. The severity of DNA damage increases proportionately with tail length and with tail type, from 1 to 4.

**Sperm analyses**

To analyze sperm number, motility and morphology, cauda epididymal sperm were released into HEPES-CZB, and incubated for at least 10 min at 37°C immediately before analysis. Sperm counts using a hemocytometer were the mean of three independent scorings per sample. For analysis of sperm morphology epididymal sperm were stained with silver nitrate as previously described (Mahadevaiah et al., 1998). Briefly, the sperm suspension (diluted as necessary with...
0.9% NaCl) was smeared on three slides, allowed to dry, fixed in methanol and acetic acid (3:1), and stained with silver nitrate. The slides were coded, and 100 sperm heads per slide were viewed at 1,000x magnification and scored. Categorization of sperm head morphology was performed as previously described (Yamauchi et al., 2009).

**Transmission electron microscope (TEM) analysis**

Cauda epididymal sperm were prepared for TEM analysis as previously described (Yamauchi et al., 2010). Briefly, samples were fixed, dehydrated, and embedded in LX-112 epoxy resin (Ladd Research Industries, Willinston, VT, USA). Ultra-thin sections (60-80 nm) were collected on Formvar-coated copper grids, double stained with uranyl acetate and lead citrate, and viewed with a LEO 912 (Zeiss, Thornwood, NY, USA) TEM. For each sample tested, 100 sperm heads were photographed at 8,000x magnification.

**CMA3 staining**

To indirectly visualize protamine-deficient DNA, CMA3 staining was applied to cryopreserved cauda epididymal sperm as previously described (Simoes et al., 2009), with modifications. Frozen-thawed sperm were washed with PBS, fixed in methanol and acetic acid (3:1, 5 min, on ice), smeared on microscope slides, and air-dried. The slides were then stained with CMA3 (0.25 mg/ml in McIlvaine buffer, pH 7.0, supplemented with 10 mM MgCl₂) for 20 min. The slides were rinsed with buffer and mounted with polyvinyl alcohol mounting medium with DABCO (Sigma, 10981) supplemented with 1.5 µg/ml DAPI. As positive controls we used sperm subjected to deproteination. To achieve deproteination sperm were incubated in 5 mM DTT, 0.5% Triton X-100 in PBS for 15 min on ice, then layered on 0.5ml of 1M sucrose in 25mM Tris-HCl, pH 7.4, centrifuged at 3,000 g for 10 min at 4°C, resuspended with methanol–glacial acetic acid (3:1) and fixed for 5 min on ice. Air-dried smears were incubated in 2M NaCl, 2 mM DTT in H₂O for 15 min at room temperature, washed thoroughly with distilled water, air-dried, and stained with CMA3, as described earlier. Microscopy images of the slides were captured using fluorescence Olympus IX81 microscope (Tokyo, Japan) with appropriate filter (460-470 nM), at 400x magnification. For analysis, at least 100 sperm heads of each sample were scored as either positive or negative, followed by measures of fluorescence intensity of each head using MetaMorph digital Imaging Software (Olympus).
Experimental design and statistical analyses

In the analysis of oocyte activation, oocyte arrest, and incidence of abnormal karyoplates, Mantel-Haenszel chi square test which allows for inter-male variability by keeping the individual male data separate was used for ‘within genotype’ comparisons and Fisher’s Exact Test was used to assess differences between the genotypes. These two tests were also used to analyze the TEM data. Expression data were analyzed with t-test. ANOVA (Generalized Linear Model as provided by NCSS statistical analysis software) with genotypes, sperm source (epididymis or testis) and sperm status (fresh or frozen) as factors was used for the analysis of comet tail lengths, oocyte activation, arrest and chromosomal integrity, and for chromosome aberration rates. The linear model ANOVA (NCSS) was also used to analyze CMA3 data in epididymal sperm. For comparison of sperm head abnormalities and of sperm motility differences between genotypes were assessed by 2-way ANOVA using the GraphPad Prism version 5.0 software. For ANOVA analyses, all percentages were transformed to angles.

The mice of interest in this study were two transgenic Sly-deficient genotypes (sh344 and sh367) and their negative siblings as controls. In all experiments except expression analyses the negative siblings of each transgenic line were compared, and if not different were pooled. In order to assess the comet, ICSI and TEM data obtained with Sly mice in the context of our earlier data from mice with NPYq deficiencies (Yamauchi et al., 2010), we first carried out ANOVA comparisons of the controls from the two studies to see if they were comparable. If this was the case, we then carried out an ANOVA analysis of the NPYq deficient and Sly-deficient genotypes compared with the pooled controls.

Acknowledgements

The authors are grateful to Paul Burgoyne who provided invaluable advice during the entire duration of the study, helped with statistical analyses, contributed to data analysis, interpretation, and drafting the manuscript. The authors also thank students M. Ward’s lab students Hisami Oda and Hieu Nguyen for help with mouse genotyping, the NIMR procedural service and O. A. Ojarikre for pronuclear injections and help with mouse breeding. The work was supported by National Institutes of Health [HD058059, RR 024206 (Project 2)] grants to MAW; JC was supported by a UK Medical Research Council Career development Fellowship [U117532009], and then by the Institut National de la Santé et de la Recherche Medicale (INSERM) and the
Fondation pour la Recherche Medicale. Microarray analyses were supported by Biotechnology and Biological Sciences Research Council (BBSRC) BB/J00877X/1 grants to PJIE.
References


Figure legends

Figure 1 - Structure of Sly gene.
A: schematic diagram of the structure of the Sly gene (GeneID: 100040308) and of its alternative splice variants: Sly1 and Sly2. The boxes numbered 1 to 10 symbolize exons, with the coding region in grey and alternatively spliced exons in hatched grey. Short hairpin RNAs (sh344 and sh367) were designed to target sequences located in exons 5 and 6 (indicated in black beneath the schematic of the Sly gene). Because exons 3-4 share ~86% of identity with exons 5-6, sh344 and sh367 can partially recognize sequences in exons 3-4 (indicated in grey beneath the schematic of the Sly gene). B: alignment of sh344 and sh367 sequences with Sly1 and with Sly2 cDNA sequence. C: Alignment of the amino acid sequence derived from exons 3-4 and exons 5-6 of Sly gene.

Figure 2 - shRNA disrupts Sly expression in sh367 and sh344 differently.
A: Sly transcripts levels in shSLY transgenic mice (tsgic, n=3 for each sh344 and sh367) and controls (neg sib, n=2 for each sh344 and sh367) obtained by real-time RT-PCR with actin as a loading control; B: Western-blot detections of SLY1 protein in testis extract from shSLY transgenic mice and controls with actin as loading control; C: Levels of SLY1 protein expression in shSLY transgenic mice (tsgic, n=4 for sh344 and n=3 for sh367) and controls (n=2 for sh344 and n=3 for sh367) quantified with ImageJ software and normalized to actin signal; D: Sly1 and Sly2 transcript levels in shSLY transgenic mice (n=3 for each sh344 and sh367) and controls (n=2 for each sh344 and sh367) obtained by semi-quantitative RT-PCR, quantified with ImageJ software and normalized to actin. In A,C&D the graphs are means ± SEM. Statistical significance with respect to corresponding control: *P<0.05, **P<0.01, ***P<0.001 (t-test). Similar results were obtained for mice on C57BL/6 and MF1 backgrounds. Neg sib = negative siblings. Primer sequences are shown in supplementary material S8.

Figure 3 - Sperm analyses in Sly-deficient mice.
The analysis was performed for two transgenic lines with Sly deficiency (sh344 and sh367) and non-transgenic siblings controls (neg sib). A: Analysis of sperm number, motility and ability to fertilize oocytes in vitro. Numbers with different superscripts within rows are statistically different (P<0.05); *
Testes from only two males were weighted. B: Sperm morphology analysis. Data shown in the Y-axis are percent of sperm analyzed. Three hundred sperm were scored per male, with number of males n=3, n=3 and n=5 for sh344, sh367, and neg sib, respectively. The X-axis represents individual categories of sperm headshapes, with N=normal, S1-S2=slight defects and G3-G8=gross defects. Examples of each headshape are shown under each sperm morphology designation. The table on the right shows significant differences between the genotypes in the proportion of specific morphology defects (2-way ANOVA). ns = non statistically significant.

**Figure 4 - Comet assay analyses of sperm from Sly-deficient mice.**

*Sly*-deficient mice, sh344 tsgic and sh367 tsgic, and their non-transgenic siblings (neg sib) were compared with distinction between the effects of genotype (A), sperm source (B; epididymal and testicular), and sperm status (C; fresh and frozen). In D, the frequency distribution of all comet tail lengths for each sperm type from neg sib and sh367 are shown.

In E-F, *Sly*-deficient mouse lines were compared to mice with moderate (2/3NPYq-) and severe (NPYq-2) NPYq deficiency. The controls here were negative siblings of shSLY mice and wild-type controls of NPYq deficient mice. In F and G, sh344 and 2/3NPYq- data are omitted due to lack of significant difference from controls. The number of males was n=8, n=10, n=3, n=5, n=2, and n=2 for neg, sib, controls, sh344 tsgic, sh367 tsgic, 2/3NPYq- and NPYq-2, respectively; 100 sperm were scored per sperm type (fresh epididymal, frozen epididymal, fresh testicular, frozen testicular) per male. Bin size: 20.

**Figure 5 - Transmission electron microscopy analysis of chromatin condensation in sperm from Sly-deficient mice.**

A-C shows examples of levels of chromatin condensation with A representing properly condensed chromatin, and B and C representing slightly (arrows) and severely decondensed chromatin, respectively. Two transgenic mouse lines with *Sly* deficiency (sh367 and sh344) and their negative siblings serving as controls were compared in single ANOVA analysis, revealing that sh367 had significantly less sperm with properly condensed chromatin than controls and sh344 (D). When *Sly*-deficient mice were put in the context of mice with moderate (2/3NPYq-) and severe (9/10NPYq- and NPYq-2), single ANOVA analysis showed a significant effect of genotype (P<0.000001), with decrease in proportion of sperm with properly condensed chromatin in order: control>2/3NPYq-=(sh344)sh367=9/10NPYq->NPYq-2 (E). Statistical
significance: a= different (P<0.05) from all other genotypes; NS = non significant. Each bar represents mean ± SEM. Number of males examined: n=7, n=3, n=5, n=7, n=13, n=3 n=2, n=3 for neg sib, sh344, sh367, control, 2/3NPYq-, 9/10NPYq-, and NPYq-2, respectively; 100 sperm heads were scored per male. Bar = 1 µm.

Figure 6 - CMA3 staining of sperm Sly-deficient and NPYq deficient mice.
A: Examples of CMA3 staining (yellow) of epididymal sperm from Sly-deficient mice (sh367) and mice with severe NPYq deficiency (9/10NPYq-); presence of CMA3 positive sperm supports that these mice have impaired sperm chromatin protamination. Positive control were sperm treated with 2mM DTT/0.5% Triton X-100 to reduce disulfide bridges and destabilize sperm chromatin. Negative control were sperm from negative siblings of sh367 mice. DAPI (blue) was used to stain nuclei. Bar = 10 µm. B: Two Sly-deficient lines (sh344 and sh367) and mice with moderate (2/3NPYq-) and severe (9/10NPYq- and NPYq-2) NPYq deficiency were compared to controls; the controls represent pooled controls specific for all genotypes. 100 sperm were scored per male, and the number of males examined per genotype was: n=27, n=9, n=6, n=3, n=6, n=6 for controls, sh344, sh367, 2/3NPYq-, 9/10NPYq-, and NPYq-2, respectively. Statistical significance: ** P<0.01; *** P<0.001 vs. control. Error = SEM.

Figure 7 - X-Y gene upregulation in Sly-deficient mice.
Gene expression in testis from two Sly-deficient lines (sh344 and sh367) was analyzed by real-time PCR. Values plotted are ΔΔCt ± standard errors. The difference in PCR cycles with respect to the spermatid specific gene Acrv1 (ΔCt), which expression is not regulated by Sly, for a given experimental sample was subtracted from the mean DCt of the reference samples (negative siblings of each transgenic line) (ΔΔCt). Three to 5 animals were analyzed per genotype. Asterisks indicate significant difference between sh344 and sh367 or between Sly-deficient mice and control values (t-test) with * P<0.05, ** P<0.01 and *** P<0.001. Primer sequences are shown in supplementary material S8.
Supplementary figure legends

Figure S1 - Transgenic SLY expression in transfected cells.
HEK293T cells were transfected with a Flag-Sly1 and Flag-Sly2 constructs and were subjected to immunostaining with an anti-FLAG antibody (green). SLY1 and SLY2 proteins were expressed, evidenced by green fluorescence. The cell nuclei are stained with DAPI (blue).

Figure S2 - Sly transcripts levels in whole testes from NPYq deficient and Sly-deficient mice.
Sly transcripts levels (A) from two Sly-deficient (shSLY) mouse lines, sh344 tsgic and sh367 tsgic and mice with moderate (2/3NPYq-) and severe (9/10NPYq- and NPYq-2) NPYq deficiency were compared by real-time RT-PCR with spermatid specific Acrv gene as a loading control. The controls were negative siblings of shSLY mice and XYRIII wild-type males; there were no differences between controls so the data from all controls were pooled. The number of males was n=3 for NPYq/Sly-deficient genotypes and n=10 for controls. In B, Sly1 and Sly2 transcript levels in whole testes from shSLY transgenic mice and 2/3NPYq- mice measured by semi-quantitative RT-PCR, quantified with ImageJ software, and normalized to spermatid specific Acrv gene are shown. The number of males was n=3 for sh344, sh367 and 2/3NPYq-, and n=6 for controls. All graphs are means ± SEM. Statistical significance (P<0.05): * vs. control, ** SLY1 vs. SLY2; columns marked with the same letter are not different from each other (t-test). Primer sequences are shown in supplementary material S8.

Figure S4 - Oocyte activation, arrest and chromosome normalcy after ICSI with sperm from Sly-deficient mice – comparison with NPYq deficient genotypes.
The data show percentages of oocytes arrested (A), oocytes with normal paternal chromosomes (B), and aberration rate reflected by total number of aberrations divided by the number of oocytes examined (C). The percentages were calculated as explained in legend to Table S3. The number of examined males was: n= 12, n=3, n=5, for control, sh344, and sh367 and n=2 for 2/3NPYq-, 9/10NPYq- and NPYq-2.

Figure S5 - Oocyte activation, arrest and chromosome normalcy after ICSI with sperm from Sly-deficient mice, preselected according to the severity of headshape defects.
Sperm originating from the same frozen-thawed cauda epididymal sperm samples from *Sly*-deficient (sh367) mice were preselected during ICSI into two groups: sperm with slight (categories S1-2 shown in Fig. 3) and gross (G4-6 shown in Fig. 3) headshape defects. The data show percentages of oocytes activated, oocytes arrested, and oocytes with normal paternal chromosomes. The percentages were calculated as explained in supplementary material S3. Sperm samples were from 4 different *Sly*-deficient males, injections were performed 6 times, and at least 37 (range 37-69) oocytes were assessed for each measured parameter per group.

**Figure S6 - Sperm nuclear protein analysis.**

To test whether increased sperm DNA damage resulted from abnormal protamination of sperm chromatin we examined cauda epididymal sperm from sh367 mice using the strategy described earlier for NPYq deficient mice. The figure shows a representative acid-urea gel separation of nuclear proteins extracted from cauda epididymal sperm corresponding to the same sperm number, and testes from *Sly*-deficient (tsgic) and non-transgenic negative siblings serving as controls (con) stained with Coomasie blue (A) and immunoblotted stain with preP2 antibody recognizing immature protamine P2 forms (B). The analysis of mean intensity of bands representing mature protamines (Coomasie and Hub2B) and immature protamines (preP2), was performed with two-way ANOVA, with gel and genotype as factors, from which the genotype means and errors were derived (C). P1 and P2 are protamine 1 and 2, respectively; PreP2 represents premature forms of protamine 2 and the antibody detecting them. Hub2B antibody recognizes mature protamine 2; the Hub2B blot is not shown due to accidental photo loss. Three independent gels were run and 5 males were tested per genotype.
A. Diagram of gene structures showing exons, introns, and shRNA locations.

B. Sequences of shRNA targets within exons 3-4 and 5-6 of Sly1 and Sly2.

C. Identity and positive scores:

Identities (*) = 23/33 (70%), Positives (+) = 26/33 (79%)

aa from exons 3-4: VKSPA FDKNENISPQAE ADEDMGDEVDSMLDKS

* +** ******* + ***** ** ********

aa from exons 5-6: VNNPAI GKDNEISPQVKGD EMGHEVGSMLDKS
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### B.

**Graph A:**
- **sh367**
- **sh344**
- **neg sib**

**Graph B:**
- **sh344**
- **sh367**

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B.  

![Bar graph showing CMA3 positive sperm (%)](bar_graph)

- Control
- 2/3NPYq−
- sh344
- tsjic
- sh367
- tsjic
- 9/10NPYq−
- NPYq-2

CMA3 positive sperm (%)

- **P** ≤ 0.01
- ***P*** ≤ 0.001
Real Time PCR quantification of X and Y transcripts

<table>
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<tr>
<th>Genes</th>
<th>sh344 tsgic</th>
<th>sh367 tsgic</th>
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<tbody>
<tr>
<td>MgcIh</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Slx</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Slx1</td>
<td>***</td>
<td>***</td>
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<tr>
<td>Actr1</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Sst1</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Sst2</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td>Asty</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Ubb</td>
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</table>

X genes

Y genes

Autosomal gene
Table 1 - Phenotype characteristics of NPYq and Sly deficient mice.

<table>
<thead>
<tr>
<th>Phenotypic effects</th>
<th>sh344</th>
<th>sh367</th>
<th>2/3NPYq-</th>
<th>9/10NPYq-</th>
<th>NPYq-2</th>
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<tbody>
<tr>
<td>Sex chromosome gene upregulation</td>
<td>*</td>
<td>***</td>
<td>**</td>
<td>***</td>
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<tr>
<td>In vivo fertility</td>
<td>=</td>
<td>***</td>
<td>*</td>
<td>****</td>
<td>****</td>
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<tr>
<td>In vitro fertility</td>
<td>=</td>
<td>***</td>
<td>**</td>
<td>****</td>
<td>****</td>
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<tr>
<td>Sperm number</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Sperm motility</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Sperm head morphology</td>
<td>=</td>
<td>**</td>
<td>*</td>
<td>***</td>
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<tr>
<td>Chromatin fragmentation in comet assay</td>
<td>=</td>
<td>*</td>
<td>=</td>
<td>***</td>
<td>****</td>
</tr>
<tr>
<td>Oocyte activation in ICSI</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>***</td>
<td>****</td>
</tr>
<tr>
<td>Oocyte arrest in ICSI</td>
<td>=</td>
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<tr>
<td>Paternal chromosome breaks in zygotes after ICSI</td>
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<tr>
<td>Chromatin condensation in TEM</td>
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<td>**</td>
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<tr>
<td>Chromatin packaging in CMA3 staining</td>
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<td>=</td>
<td>***</td>
<td>****</td>
</tr>
</tbody>
</table>

Estimated levels of impairment: = not affected; * slightly affected; ** moderately affected; *** severely affected **** very severely affected. Shaded part of the table shows phenotypic effects associated with sperm chromatin packaging/DNA damage. The levels of phenotypic effects of NPYq/Sly- deficient mice presented in this table are based on the present study and on our unpublished data, as well as on several previously published manuscripts from our group and few others (Cocquet et al., 2009; Ellis et al., 2005; Grzmił et al., 2007; Reynard et al., 2007; Styrna et al., 2002; Styrna et al., 2003; Toure et al., 2005; Toure et al., 2004b; Ward and Burgoyne, 2006; Xian et al., 1992; Yamauchi et al., 2010; Yamauchi et al., 2009) and therefore should be considered an estimate.